

Application
for
United States Letters Patent

To all whom it may concern:

Be it known that WEI, Guangwen, GUO, Rongbing and ZHANG,
Renhuai

have invented certain new and useful improvements in

RECOMBINANT SUPER-COMPOUND INTERFERON

of which the following is a full, clear and exact
description

RECOMBINANT SUPER-COMPOUND INTERFERON

The application is a continuation-in-part application of International Patent Application No. PCT/CN02/00128, filed 5 on 28 February 2002, which claims priority of Chinese Application No. 01104367.9, filed on 28 February 2001, the contents of which are incorporated by reference here into this application.

10 Throughout this application, various references are referred to. Disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

15

FIELD OF THE INVENTION

This invention is related to a recombinant super-compound interferon (rSIFN-co) with changed spatial configuration. One characteristic of rSIFN-co in this invention is that it 20 cannot only inhibit DNA (deoxyribonucleic acid) duplication of the hepatitis B virus but also the secretion of HBsAg and HBeAg.

25 **BACKGROUND OF THE INVENTION**
rSIFN-co is a new interferon molecule constructed with the most popular conservative amino acid found in natural human α -IFN subtypes using genetic engineering methods. United States Patent Nos. 4,695,623 and 4,897,471 have described it. rSIFN-co had been proved to have broad-spectrum IFN 30 activity and virus- and tumor-inhibition and natural killer cell activity. United States Patent No. 5,372,808 by Amgen, Inc. addresses treatment rSIFN-co. Chinese Patent No. 97193506.8 by Amgen, Inc. addresses re-treatment of rSIFN-co on hepatitis C. Chinese Patent No. 98114663.5 by 35 Shenzhen Jiusheng Bio-engineering Ltd. addresses treatment of rSIFN-co on hepatitis B and hepatitis C.

The United States Food and Drug Administration (FDA)

authorized Amgen to produce rSIFN-co with *E. Coli.* for clinical hepatitis C treatment at the end of 1997.

Hepatitis B patients can be identified when detecting HBsAg and the HBeAg. α -IFN is commonly used in clinics to treat hepatitis B. IFN binds superficial cell membrane receptors, inhibiting DNA and RNA (ribonucleic acid) duplication, including inducing some enzymes to prevent duplication of the virus in hepatitis-infected cells. All IFNs can inhibit only the DNA duplication of viruses, not the e and s antigen.

This disclosure describes recombinant super-compound interferon, method to produce the same and uses thereof.

SUMMARY OF THE INVENTION

This invention provides a recombinant super-compound interferon or an equivalent thereof with changed spatial configuration. An equivalent is a molecule which is similar in function to the super-compound interferon. The super-compound interferon possesses anti-viral or anti-tumor activity. This invention also provides an artificial gene codes for the super-compound interferon or its equivalent.

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This invention provides a process for production of recombinant super-compound interferon comprising introducing an artificial gene with selected codon preference into an appropriate host, culturing said introduced host in an appropriate condition permitting expression of said super-compound interferon and harvesting the expressed super-compound interferon.

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This invention provides a composition comprising the recombinant super-compound interferon or its equivalent and a suitable carrier. This invention further provides a pharmaceutical composition comprising the recombinant super-compound interferon or its equivalent and a pharmaceutically acceptable carrier.

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This invention provides a method for treating viral diseases or tumor in a subject comprising administering to the subject an effective amount of the super-compound interferon or its equivalent.

35

This invention provides the above-described method wherein super-compound interferon was administered via oral, vein injection, muscle injection, peritoneal injection, subcutaneous injection, nasal, mucosal administration, by inhalation via an inspirator.

DETAILED DESCRIPTION OF THE FIGURES

5 **Figure 1.** rSIFN-co cDNA sequence designed according to *E.*
Coli. codon usage and deduced rSIFN-co amino acid sequence

Figure 2. Sequence of another super-compound interferon

10 **Figure 3.** Diagram of pLac T7 cloning vector plasmid

Figure 4. Diagram of pHY-4 expression vector plasmid

Figure 5. Construction process of expression plasmid pHY-5

15 **Figure 6-A.** Circular Dichroism spectrum of Infergen[®]
Spectrum range: 250nm - 190nm

Sensitivity: 2 m°/cm

Light path: 0.20 cm

Equipment: Circular Dichroism J-500C

20 Samples: contains 30µg/ml IFN-con1, 5.9 mg/ml of NaCl and
3.8 mg/ml of Na₂PO₄, pH7.0.

25 Infergen[®] (interferon alfacon-1), made by Amgen Inc., also
known as consensus interferon, is marketed for the
treatment of adults with chronic hepatitis C virus (HCV)
infections. It is currently the only FDA approved, bio-
optimized interferon developed through rational drug design
and the only interferon with data in the label specifically
for non-responding or refractory patients. InterMune's
30 sales force re-launched Infergen[®] in January 2002 with an
active campaign to educate U.S. hepatologists about the
safe and appropriate use of Infergen[®], which represents new
hope for the more than 50 percent of HCV patients who fail
other currently available therapies. See
35 <http://www.intermune.com/wt/itmnn/infergen>, 8/27/2003

Figure 6-B. Circular Dichroism spectrum of Infergen® From Reference [Journal of Interferon and Cytokine Research. 16:489-499 (1996)]

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Figure 6-C. Circular Dichroism spectrum of rSIFN-co
Spectrum range: 320nm-250nm
Sensitivity: 2 m°/cm
Light path: 2cm
10 Equipment: Circular Dichroism J-500C
Samples: contains 0.5mg/ml rSIFN-co, 5.9 mg/ml of NaCl and 3.8 mg/ml of Na₂PO₄, pH7.0.

Figure 6-D. Circular Dichroism spectrum of rSIFN-co
15 Spectrum range: 250nm - 190nm
Sensitivity: 2 m°/cm
Light path: 0.20 cm
Equipment: Circular Dichroism J-500C
20 Samples: contains 30μg/ml rSIFN-co, 5.9 mg/ml of NaCl and 3.8 mg/ml of Na₂PO₄, pH7.0.

Clearly, as evidenced by the above spectra, the secondary or even tertiary structure of rSIFN-co is different from Infergen®.

25

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a recombinant super-compound interferon or an equivalent thereof with changed spatial configuration. This invention reveals that protein with same primary sequence might have different biological activities. As illustrated in the following example, this invention disclosed two proteins with identical amino acid sequence but with different activities. This activity may sometimes become improved efficacy and sometimes, the protein with changed spatial configuration would reveal new function.

An equivalent is a molecule which is similar in function to the compound interferon. An equivalent could be a deletion, substitution, or replacement mutant of the original sequence. Alternatively, it is also the intention of this invention to cover mimics of the recombinant super-compound interferon. Mimics could be a peptide, polypeptide or a small chemical entity.

The interferon described herein includes but is not limited to interferon α , β , or ω . In an embodiment, it is IFN-1a, IFN-2b or other mutants.

In an embodiment, the super-compound interferon disclosed has higher efficacy than the interferon described in U.S. Patent Nos. 4,695,623 or 4,897,471. This super-compound interferon is believed to have unique secondary or tertiary structure. (See e.g. Figure 6)

The super-compound interferon described herein has spatial structure change(s) resulting from the changes of its production process.

The above-described super-compound interferon may be produced by a high efficiency expression system which uses

a special promoter. In an embodiment, the promoter is P_{BAD}. As it could be easily appreciated by other ordinary skilled artisan, other inducible promoter, such as heat shock promoter, may be used in this invention.

5

The super-compound interferon may also be produced with its gene as artificially synthesized cDNA with adjustment of its sequence from the wild-type according to codon preference of *E. Coli*. Extensive discussion of said codon usage (preference) may be found in U.S. Patent No. 10 4,695,623. See e.g. column 6, line 41 - column 7, line 35

15 The above described super-compound interferon possesses anti-viral or anti-tumor activity and therefore useful in preventing and treating viral diseases, tumors or cancers.

20 The virus diseases include but are not limited to hepatitis A, hepatitis B, hepatitis C, other types of hepatitis, infections caused by Epstein-Barr virus, Cytomegalovirus, herpes simplex viruses, other herpes viruses, papovaviruses, poxviruses, picornaviruses, adenoviruses, rihnoviruses, human T cell leukaemia viruses I, human T cell leukaemia viruses II, or human T cell leukemia viruses III.

25

Therefore, this invention provides a method for inhibiting virus replication or virus infected cells by contacting said virus or infected cells with an effective amount of the super-compound interferon or its equivalent. This super-compound interferon is useful in preventing or treating the following cancers or tumors:

Cancer	Skin Cancer	Basal Cell Carcinoma
		Malignant Melanoma
	Renal cell carcinoma	
	Liver Cancer	

Malignant Hemal Disease	Thyroid Cancer	
	Rhinopharyngeal Cancer	
	Solid Carcinoma	Prostate Cancer Tummy Cancer Esophagus Cancer Recta Cancer Pancreas Cancer Mammary Cancer
	Ovarian Cancer & Superficial Bladder Cancer	
	Hemangioma	
	Epidermoid Carcinoma	Cervical Cancer Non-small Cell Lung Cancer Small Cell Lung Cancer Glioma
	Leucocytethmia	Acute Leucocytethmia Chronic Leucocytethmia
	Chronic Myelocytic Leukemia	
	Hairy Cell Leukemia	
	Lymphadenoma	
	Multiple Myeloma	
	Polycythemia Vera	
	Kaposi's Sarcoma	
Others		

Accordingly, this invention provides a method for inhibiting tumor or cancer cell growth by contacting the super-compound interferon or its equivalent with said tumor or cancer cells. In a further embodiment, the super-

compound interferon inhibits the DNA duplication and secretion of HBsAg and HBeAg of Hepatitis B Virus.

This invention also provides an artificial gene codes for
5 the super-compound interferon or its equivalent. It is
within the ordinary skill to design an artificial gene.
Many methods for generating nucleotide sequence and other
molecular biology techniques have been described previously.
See for example, Joseph Sambrook and David W. Russell,
10 Molecular Cloning: A laboratory Manual, December 2000,
published by Cold Spring Harbor Laboratory Press.

This invention provides a vector comprising the gene which
codes for the super-compound interferon or its equivalent.

15 This invention provides an expression system comprising the
vector comprising the gene which codes for the super-
compound interferon or its equivalent. The cells include
but are not limited to prokaryotic or eukaryotic cells.

20 This invention also provides a host cell comprising the
vector comprising the gene which codes for the super-
compound interferon or its equivalent.

25 This invention provides a process for production of
recombinant super- compound interferon comprising
introducing an artificial gene with selected codon
preference into an appropriate host, culturing said
introduced host in an appropriate condition for the
expression of said compound interferon and harvesting the
30 expressed compound interferon.

The process may comprise extraction of super-compound
interferon from fermentation broth, collection of inclusion
35 body, denaturation and renaturation of the harvested
protein.

- The process may maintain the high efficacy even when the super-compound interferon is used with an agent and in a particular concentration. The process also comprises separation and purification of the super-compound interferon. The process further comprises lyophilization of the purified super-compound interferon. The process comprises production of liquid injection of super-compound interferon.
- This invention also provides the produced super-compound interferon by the above processes.
- This invention provides a composition comprising the recombinant super-compound interferon or its equivalent and a suitable carrier.
- This invention provides a pharmaceutical composition comprising the recombinant super-compound interferon or its equivalent and a pharmaceutically acceptable carrier.
- This invention provides a method for treating viral diseases or tumor in a subject comprising administering to the subject an effective amount of the super-compound interferon or its equivalent.
- This invention provides the above-described method wherein the viral diseases is hepatitis A, hepatitis B, hepatitis C, other types of hepatitis, infections of viruses caused by Epstein-Barr virus, Cytomegalovirus, herpes simplex viruses, or other type of herpes viruses, papovaviruses, poxviruses, picornaviruses, adenoviruses, rihnoviruses, human T cell leukaemia viruses I, or human T cell leukaemia viruses II, or human T cell leukemia virus III.
- This invention provides the above-described method wherein super-compound interferon was administered via oral, vein

injection, muscle injection, peritoneal injection, subcutaneous injection, nasal, mucosal administration, by inhalation via an inspirator.

5 This invention provides the above-described method wherein super-compound interferon was administered following the protocol of injection 9 μ g or 15 μ g per day, 3 times a week, total 24 weeks.

10 It was surprising to find that rSIFN-co, the spatial structure of which has been changed, is not only a preparation to inhibit the DNA duplication of hepatitis B, but to inhibit the secretion of HBsAg and HBeAg on 2.2.15 cells.

15 One objective of this invention is to offer a preparation of rSIFN-co to directly inhibit the DNA duplication of hepatitis B viruses and the secretion of HBeAg and HBsAg of hepatitis B and decrease them to normal levels.

20 In one of the results of this invention, rSIFN-co was produced with recombinant techniques. On the condition of fixed amino acid sequence, the IFN DNA was redesigned according to the *E. Coli.* codon usage and then the rSIFN-co gene was artificially synthesized. rSIFN-co cDNA was cloned into the high-expression vector of *E. Coli.* by DNA recombinant techniques, and a high expression of rSIFN-co was gained by using of induce/activate-mechanism of L-arabinose to activate the transcription of P_{BAD} promoter.

25 30 Compared with usual thermo-induction, pH induction and IPTG induction systems of genetic engineering, arabinose induction/activation system has some advantages: (1) Common systems relieve promoter function by creating a "derepression" pattern. Promoters then induce downstream gene expression. So temperature and pH change and the addition of IPTG cannot activate promoters directly. In the system disclosed herein, L-arabinose not only deactivates

- and represses but also activates the transcription of P_{BAD} promoter which induce a high expression of rSIFN-co. Therefore, the arabinose induction/activation system is a more effective expression system. (2) The relation between 5 Exogenous and L-arabinose dosage is linearity. This means the concentration of arabinose can be changed to adjust the expression level of the exogenous gene. Therefore, it is easier to control the exogenous gene expression level in *E. Coli.* by arabinose than by changing temperature and pH 10 value. This characteristic is significant for the formation of inclusion bodies. (3) L- arabinose is resourceful cheap and safe, which, on the contrary, are the disadvantages of other inducers such as IPTG.
- 15 This embodiment creates an effective and resistant rSIFN-co-expressing *E. Coli.* engineering strain with an L-arabinose induction/activation system. The strain is cultivated and fermented under suitable conditions to harvest the bacterial bodies. Inclusion bodies are then 20 purified after destroying bacteria and washing repeatedly. The end result, mass of high-purity, spatial-configuration-changed rSIFN-co protein for this invention and for clinical treatment, was gained from denaturation and renaturation of inclusion bodies and a series of 25 purification steps.
- The following are some rSIFN-co preparations: tablets, capsules, oral liquids, pastes, injections, sprays, suppositories, and solutions. Injections are recommended. 30 It is common to subcutaneously inject or vein-inject the medicine. The medicine carrier could be any acceptance medicine carrier, including carbohydrate, cellulose, adhesive, collapse, emollient, filling, add-dissolve agent, amortization, preservative, add-thick agent, matching, etc.
- 35 This invention also provides a pharmaceutical composition comprising the above composition and a pharmaceutically

acceptable carrier.

For the purposes of this invention, "pharmaceutically acceptable carriers" means any of the standard pharmaceutical carriers. Examples of suitable carriers are well known in the art and may include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution and various wetting agents. Other carriers may include additives used in tablets, granules and capsules, etc. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gum, glycols or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well-known conventional methods.

This invention will be better understood from the examples which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

25 EXPERIMENTAL DETAILS

EXAMPLE 1

rSIFN-co is a new interferon molecule constructed according to conservative amino acid in human IFN- α subtype with 30 genetic engineering method. It has been proven that rSIFN-co has broad-spectrum IFN activity, such as high antiviruse and tumor inhibition activity, especially for effectively treating hepatitis C.

35 E. Coli. codon was used to redesign rSIFN-co cDNA and then artificially synthesize cDNA of rSIFN-co from published rSIFN-co DNA sequences and deduced amino acid sequences

(Figure 1).

In order to get pure rSIFN-co protein, rSIFN-co cDNA was cloned into E. Coli. high-expression vector, and L-5 arabinose, which can activate strong P_{BAD} promoter in vectors, was used to induce high expression of rSIFN-co gene.

Synthesis of E. Coli. cDNA Sequence

Redesign of rSIFN-co cDNA sequence

10 rSIFN-co cDNA was redesigned according to the codon usage of E. Coli. to achieve high expression in E. Coli. Deduced amino acid sequence from the redesigned cDNA sequence of rSIFN-co is completely coincidental with primitive amino acid sequence of published rSIFN-co (Figure 1).

rSIFN-co cDNA sequence synthesis

rSIFN-co cDNA 5'-terminus and 3'- terminus semi-molecular synthesis

Two semi-molecules can be directly synthesized: rSIFN-co cDNA 5'- terminus 280bp (fragment I) and 3'- terminus 20 268bp(fragment II) by PCR. There are 41bp overlapping among fragment II and fragment I.

(1) Chemical synthesis oligodeoxynucleotide fragment:

Oligomer A:

25 5' ATGTGCGACCTGCCGCAGACCCACTCCCTGGGTAACCGTCGTGCTCTGATCCTGCTGGCTCA
GATGCGTCGTATCTCCCCGTTCTCCTGCCTGAAAGACCGTCACGAC3'

Oligomer B:

5' CTGAAAGACCGTCACGACTTCGGTTCCCGCAGGAGAGGTTCGACGGTAACCAGTTCCAGA
AGCTCAGGCTATCTCCGTTCTGCACGAAATGATCCAGCAGACCTTC3'

30 Oligomer C:

5' GCTGCTGGTACAGTTGGTGTAGAATTTCAGCAGGGATTGCTCCAAAGCAGCGGAGGAG
TCTTTGGTGGAGAACAGGTTGAAGGTTGCTGGATCATTTC3'

Oligomer D:

5' ATCCCTGCTGGAAAAATTCTACACCGAACTGTACCAGCAGCTGAACGACCTGGAAGCTTGCG

TTATCCAGGAAGTTGGTGTGAAGAAACCCGCTGATGAAC3'

Oligomer E:

5' GAAGAAACCCCGCTGATGAACGTTGACTCCATCCTGGCTGTTAAAAAAACTTCCAGCGTAT
CACCCCTGTACCTGACCGAAAAAAAATACTCCCCGTGCGCTTGGG3'

5 Oligomer F:

5' TTATTCTTTACGACGCAGACGTTCCTGCAGGTTGGACAGGGAGAAGGAACGCATGATT
CAGCACGAACAACTCCCAAGCGCACGGGGAGTATTTTCGGTCAGG3'

10 PCR I for Fragment I: oligodeoxynucleotide B as template,
oligodeoxynucleotide A and C as primers, synthesized 280 bp
Fragment I.

PCR I mixture (units: μ l)

sterilized distilled water	39
10×Pfu buffer (Stratagen American Ltd.)	5
dNTP mixture (dNTP concentration 2.5 mmol/L)	2
Oligomer A primer (25 μ mol/L)	1
Oligomer C primer (25 μ mol/L)	1
Oligomer B template (1 μ mol/L)	1
Pfu DNA polymerase (Stratagen American Ltd.) (25 U/ μ l)	1
Total volume	50 μ l

PCR cycle: 95 I

15

2m→(95°C45s→65°C1m→72°C1m)×25 cycle→72°C10m→4°C

20 PCR II for Fragment II: oligodeoxynucleotide E as
template, oligodeoxynucleotide D and F as primers,
synthesized 268bp Fragment II.

PCR II mixture (units: μ l)

sterilized distilled water	39
10×Pfu buffer (Stratagen American Ltd.)	5
dNTP mixture (dNTP concentration 2.5mmol/L)	2
Oligomer D primer (25 μ mol/L)	1
Oligomer F primer (25 μ mol/L)	1
Oligomer E template (1 μ mol/L)	1

Pfu DNA polymerase (Stratagen American Ltd.) (25U/ μ l) 1
Total volume 50 μ l
PCR cycle: the same as PCR I

Assembling of rSIFN-co cDNA

5 Fragment I and II were assembled together to get the complete cDNA molecular sequence of rSIFN-co using the overlapping and extending PCR method. Restriction enzyme Nde I and Pst I were introduced to clone rSIFN-co cDNA sequence into plasmid.

10 (1) Chemical synthesis primers

Oligomer G: 5'ATCGGCCATATGTGCGACCTGCCGCAGACCC3'

Oligomer H: 5'ACTGCCAGGCTGCAGTTATTCTTACGACGCAGACGTTCC3'

(2) Overlapping and extending PCR

PCR mixture	(units: μ l)
sterilized distilled water	38
10×Pfu buffer (Stratagen American Ltd.)	5
dNTP mixture (dNTP concentration 2.5mmol/L)	2
primer G (25 μ mol/L)	1
primer H (25 μ mol/L)	1
*fragment I production (1 μ mol/L)	1
*fragment II production (1 μ mol/L)	1
Pfu DNA polymerase (Stratagen American Ltd.) (2.5U/ μ l)	1
Total volume	50 μ

*Separate and purify PCR production with StrataPrep PCR purification kit produced by Stratagen American Ltd. And dissolve into sterilized distilled water.

20 PCR cycle: the same as PCR I

rSIFN-co gene clone and sequence analysis

pLac T7 plasmid as cloning vector. pLac T7 plasmid is reconstructed with pBluescript II KS(+) plasmid produced by Stratagen (Figure 3).

- Purified PCR production of rSIFN-co cDNA with StrataPrep PCR purification kit. Digest cDNA and pLac T7 plasmid with NdeI and PstI. Run 1% agarose gel electrophoresis and separate these double-digested DNA fragments. Recover 507bp
5 long rSIFN-co DNA fragment and 2.9kb plasmid DNA fragment. Ligate these fragments by T4 DNA ligase to form a recombinant plasmid. Transform DH₅α competent cells (Gibco) with the recombinant plasmid, culture at 37°C overnight. Identify the positive recombinant colony, named pHY-1.
- 10 Run DNA sequencing with SequiTHERM™ Cycle Sequencing Kit produced by American Epicentre Technologies Ltd using L1-COR Model 4000L. Primers are T7 and T3 common sequence primer, the DNA sequencing result matches theoretic design.
- 15 Purify the rSIFN-co, sequence the N-terminus amino acids, the N-terminus amino acid sequence matches experimental design which is as follows:
- N- Cys-Asp-Leu-Pro-Gln-Thr-His-Ser-Leu-Gly-Asn-Arg-Arg-Ala-Leu-
- 20 **Construction, transformation, identification, and hereditary stability of expression vector**
- Construction and transformation of expression vector**
- Digested E. Coli. expression vector pHY-4 (see Figure 3) with Nde I to linearize and subsequently digest with Xba I.
25 Run 1% agarose gel electrophoresis, and purify the 4.8kb pHY-4 Nde I -Xba I digest fragment with QIAEX II kit produced by QIAGEN Germany Ltd.
- At the same time, the pHY-4 plasmid is double digested with Nde I-Xba I. Run 1% agarose gel electrophoresis and purify the 715bp fragment. Ligate the rSIFN-co and pHY-4 fragments with T4 DNA ligase to construct the recombinant plasmid
30 (See Figure 4). Transform DH₅α competent cells with the

recombinant plasmid. Spread the transformed cells on LB plate with Amp, 37°C culture overnight.

Positive cloning strain screening

5 Randomly choose *E. Coli.* colonies from above LB-plate, screening the positive strains containing recombinant vector by endonuclease digesting and PCR analysis. Name one of the positive recombinant plasmid pHY-5, and name the strain containing pHY-5 plasmid PVIII. Amplify and store the positive strain with glycerol in -80°C.

10 **High expression of rSIFN-co gene in *E. Coli.***

In pHY-5 plasmid, rSIFN-co gene is under control of strong promoter P_{BAD} . This promoter is positively and negatively regulated by the product of the gene araC. AraC is a transcriptional regulator that forms a complex with arabinose. In the absence of arabinose, the AraC dimer binds O₂ and I₁ forming a 210bp loop. This conformation leads to a complete inhibition of transcription. In the presence of arabinose, the dimer is released from O₂ and binds I₁ and I₂, leading to transcription. Arabinose binding deactivates, represses and even activates the transcription of P_{BAD} promoter, which stimulates P_{BAD} inducing high expression of rSIFN-co. rSIFN-co expression level in PVIII is more than 50% of the total *E. Coli.* protein.

15 **Summary**

25 RSIFN-CO is a new interferon molecule artificially built according to the conservative amino acid of human α interferons. It has been proven as a effective anti-hepatitis drug. In order to get enough pure rSIFN-co protein, a stable recombinant *E. Coli.* strain which high expresses rSIFN-co protein was constructed.

30 First, according to published rSIFN-co amino acid sequence,

E. Coli. codon was used to synthesize whole cDNA of rSIFN-co. This DNA fragment was sequenced and proved that the 501bp codon sequence and TAA termination codon sequence are valid and identical with theocratic design. Subsequent 5 analysis revealed that the N-terminus amino acid sequence and amino acid composed of rSIFN-co produced by the recombinant strain were both identical to the prediction.

The rSIFN-co cDNA was cloned into E. Coli. high-expression 10 vector pHY-4 plasmid to construct the recombinant plasmid pHY-5. E. Coli. LMG194 strain was further transformed with pHY-4 plasmid to get stable rSIFN-co high-expression transformant. This transformant was cultured for 30 generations. The heredity of pHY-5 recombinant plasmid in E. 15 Coli. LMG194 was normal and stable, and the expression of rSIFN-co was high and steady.

E. Coli. LMG194, which contains recombinant pHY-5 plasmid, is actually an ideal high-expression engineering strain.

20 **References**

1. Blatt LM, Davis JM, Klein SB. et al. The biologic activity and molecular characterization of a novel synthetic interferon-alpha species, consensus interferon. Journal of Interferon and Cytokine Research, 1996;16(7):489-499.
2. Alton,K.et al: Production characterization and biological effects of recombinant DNA derived human IFN- α and IFN- γ analogs. In: De Maeger E, Schellekens H. eds. The Biology of Interferon System.2nd ed. Amsterdam: Elsevier 30 Science Publishers, 1983: 119-128
3. Pfeffer LM. Biologic activity of natural and synthetic type 1 interferons. Seminars in Oncology, 1997;24 (3 suppl 9):S9-63--S9-69.
4. Ozes ON, Reiter Z, Klein S, et al. A comparison of interferon-con1 with natural recombinant interferons-(: 35

- antiviral, antiproliferative, and natural killer-inducing activities. J. Interferon Res., 1992; 12:55-59.
5. Heathcote EJL, Keeffe EB, Lee SS, et al. Re-treatment of chronic hepatitis C with consensus interferon. Hepatology, 1998;27(4):1136-1143.
6. Klein ML, Bartley TD, Lai PH, et al. Structural characterization of recombinant consensus interferon-alpha. Journal of Chromatography, 1988; 454:205-215.
7. The Wisconsin Package, by Genetics Computer Group, Inc.
10 Copyright 1992, Medison, Wisconsin, USA
8. Nishimura, A et al: A rapid and highly efficient method for preparation of competent E. coli cells. Nuclei. Acids Res. 1990, 18:6169
9. All molecular cloning techniques used are from: Sambrook, J., E. F. Fritsch and T. Maniatis. Molecular Cloning:A laboratory manual, 2nd ed. CSH Laboratory Press, Cold Spring Harbour, NY.1989.
10. Guzman, L. M et al: Tight regulation, modulation, and high-level expression by vectors containing the arabinose
15 PBAD promoter. J. Bacteriol. 1995, 177: 4121~ 4130.
- 20

rSIFN-co cDNA SEQUENCE DESIGNED ACCORDING TO E. COLI. CODON USAGE AND DEDUCED rSIFN-co AMINO ACID SEQUENCE

25 5' 11 21 31 41 51
+1 M C D L P Q T H S L G N R R A L I L L A
1 ATGTGCGACC TGCCGCAGAC CCACTCCCTG GGTAACCGTC GTGCTCTGAT CCTGCTGGCT
TACACGCTGG ACGGCGTCTG GGTGAGGGAC CCATTGGCAG CACGAGACTA GGACGACCGA

30 5' 71 81 91 101 111
+1 Q M R R I S P F S C L K D R H D F G F P
61 CAGATGCGTC GTATCTCCCC GTTCTCCTGC CTGAAAGACC GTCACGACTT CGGTTCCCG
GTCTACGCAG CATAGAGGGG CAAGAGGACG GACTTCTGG CAGTGCTGAA GCCAAAGGGC

35 5' 131 141 151 161 171
+1 Q E E F D G N Q F Q K A Q A I S V L H E
121 CAGGAAGAAT TCGACGGTAA CCAGTTCCAG AAAGCTCAGG CTATCTCCGT TCTGCACGAA

GTCCTTCTTA AGCTGCCATT GGTCAAGGTC TTTCGAGTCC GATAGAGGCA AGACGTGCTT

5 5' 191 201 211 221 231
+1 M I Q Q T F N L F S T K D S S A A W D E
181 ATGATCCAGC AGACCTTCAA CCTGTTCTCC ACCAAAGACT CCTCCGCTGC TTGGGACGAA
TACTAGGTCTG TCTGGAAGTT GGACAAGAGG TGTTTCTGA GGAGGCGACG AACCTGCTT

10 5' 251 261 271 281 291
+1 S L L E K F Y T E L Y Q Q L N D L E A C
241 TCCCTGCTGG AAAAATTCTA CACCGAACTG TACCAAGCAGC TGAACGACCT GGAAGCTTGC
AGGGACGACC TTTTTAAGAT GTGGCTTGAC ATGGTCGTG ACITGCTGGA CCTTCGAACG

15 5' 311 321 331 341 351
+1 V I Q E V G V E E T P L M N V D S I L A
301 GTTATCCAGG AAGTTGGTGT TGAAGAAAACC CCGCTGATGA ACGTTGACTC CATCCTGGCT
CAATAGGTCC TTCAACCACA ACTTCTTGG GGCAGACTACT TGCAACTGAG GTAGGACCGA

20 5' 371 381 391 401 411
+1 V K K Y F Q R I T L Y L T E K K Y S P C
361 GTTAAAAAAAT ACTTCCAGCG TATCACCCCTG TACCTGACCG AAAAAAAAATA CTCCCCGTGC
CAATTTTTA TGAAGGTGCG ATAGTGGAC ATGGACTGGC TTTTTTTAT GAGGGGCACG

25
5' 431 441 451 461 471
+1 A W E V V R A E I M R S F S L S T N L Q
421 GCTTGGGAAG TTGTTCTGTC TGAAATCATG CGTTCCCTCT CCCTGTCCAC CAACCTGCAG
CGAACCCCTTC ACAAGCACG ACTTTAGTAC GCAAGGAAGA GGGACAGGTG GTTGGACGTC

30
5' 491 501
+1 E R L R R K E #
481 GAACGTCTGC GTCGTAAAGA ATAA
CTTGCAGACCG CAGCATTCT TATT

35
EXAMPLE 2
Separation and purification of rSIFN-co
1. Fermentation
Inoculate the recombinant strain in LB media, shaking (200

rpm) under 37°C overnight (approximate 18 h), then add 30% glycerol to the fermentation broth to get final concentration of 15%, allotted to 1 ml tube and kept in -20°C as seed for production.

5

Add 1% of the seed to LB media, shaking (200 rpm) under 37°C overnight to enlarge the scale of the seed, then add to RM media with a ratio of 10%, culturing under 37°C. Add arabinose (20% solution) to 0.02% as an inductor when the OD600 reaches about 2.0. 4 hours after that, stop the culture process, collect the bacteria by centrifuge, resuspend the pellet with buffer A, and keep in -20°C overnight. Thaw and break the bacteria by homogenizer, then centrifuge. Wash the pellet with buffer B, buffer C, and distilled water to get a relatively pure inclusion body.

10

15

2. Denaturation and renaturation

20

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Dissolve the inclusion body in Guanidine-HCl (or urea) of 6 mol/L. The solution will be a little cloudy. Centrifuge it at a speed of 10000 rpm. Determine the protein concentration of the supernatant. This supernatant is called "denaturation solution." Add the denaturation solution to renaturation buffer, and keep the final protein concentration under 0.3 mg/ml. It is better to add the totally denaturation solution in three steps instead of one step. Keep the solution overnight under 4°C. Afterwards, dialyze 10 mol/L, 5 mol/L PB buffer and distilled water, then adjust its pH by 2 mol/L HAc-NaAc. Let it stand, then filtrate.

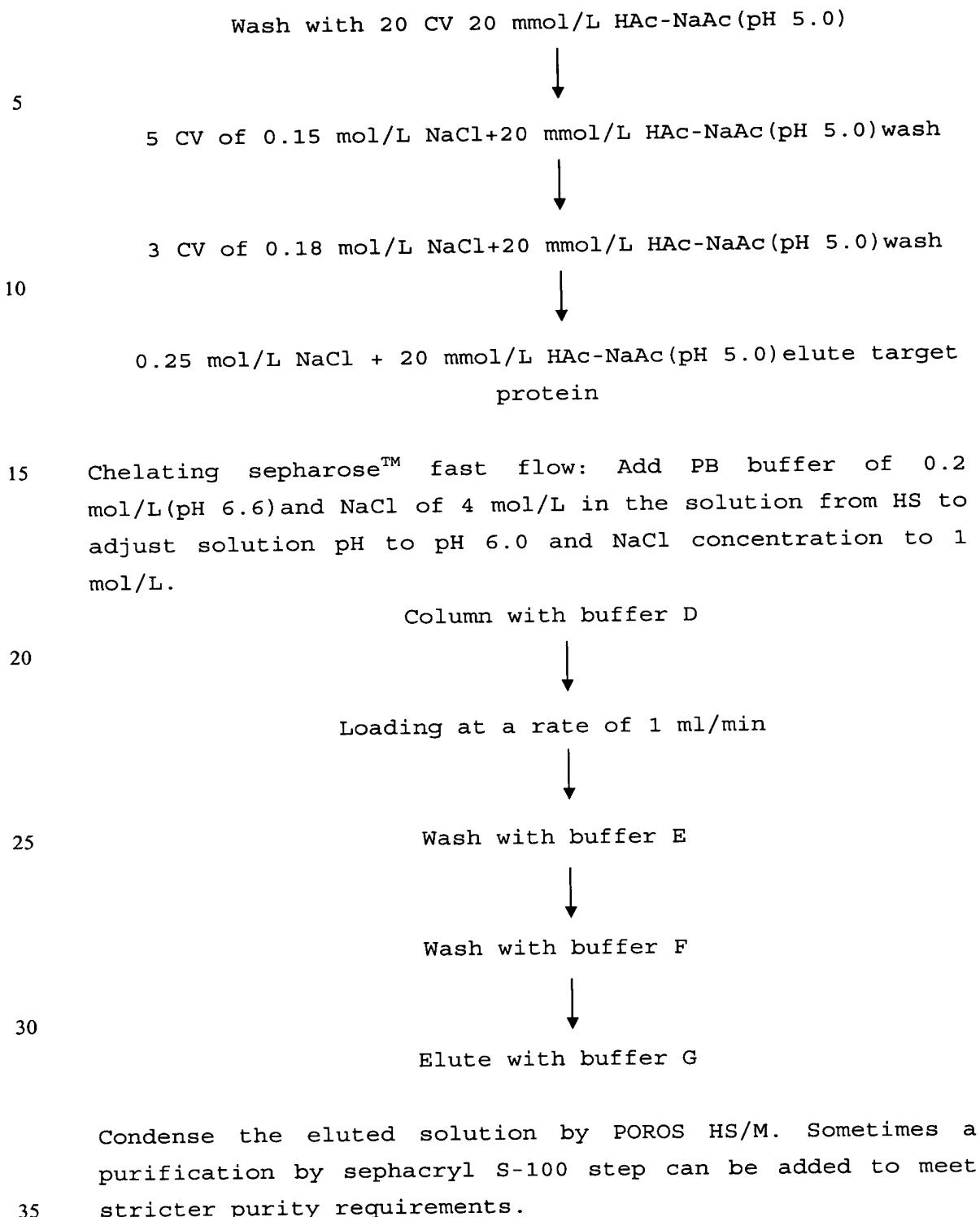
3. Purification

POROS HS/M anion exchange chromatography:

Equivalent column with 20 mmol/L HAc-NaAc (pH 5.0)

35

Load samples at a speed of 30 ml/min



Note:

Buffer A: 100 mmol/L Tris-HCl, pH 7.5-10 mmol/L EDTA-100 mmol/L NaCl

5 Buffer B: 50 mmol/L Tris-HCl, pH 7.5-1 mol/L Urea-10 mmol/L EDTA-0.5% Triton X-100

Buffer C: 50 mmol/L Tris-HCl, pH 7.5-2 mol/L Urea-10 mmol/L EDTA-0.5% Triton X-100

Buffer D: 1 mol/L NaCl ---50 mmol/L Na₂HPO₄ (pH 5.5)

Buffer E: 1 mol/L NaCl ---50 mmol/L Na₂HPO₄ (pH 5.0)

10 Buffer F: 1 mol/L NaCl ---50 mmol/L Na₂HPO₄ (pH 4.0)

Buffer G: 1 mol/L NaCl ---50 mmol/L Na₂HPO₄ (pH 3.6)

Renaturation buffer: 0.5 mol/L Arginine-150 mmol/L Tris-HCl, pH 7.5-0.2 mmol/L EDTA

LB Media: 1 L

15 Tryptone 10 g

Yeast extracts 5 g

NaCl 10 g

RM Media: 1 L

Casein 20 g

20 MgCl 1 mmol/L (0.203 g)

Na₂HPO₄ 4 g;

KH₂PO₄ 3 g,

NaCl 0.5 g

NH₄Cl 1 g

25

After purification, the buffer was changed to PBS (pH 7.0) along with the step of condensing by POROS HS/M. This is called the "Protein Stock Solution." It can directly used in the preparation of injections or sprays, or stored at 2-30 8°C.

Formula for injection:

	Solution	Lyophilized powder
Solution of rSIFN-	34.5 µg/ml	34.5 µg/ml
CO		
PB (pH7.0)	25mmol/L	10mmol/L

Glycine	---	0.4mol/L
NaCl	0.1mol/L	-----

For spray:

EDTA	0.01%
Tween 80	0.05%
Trisodium citrate	10mmol/L
Glycerol	1.26%
Sodium Chloride	0.03%
Phenylmethanol	0.5%
HSA	0.1%
rSIFN-co	10 µg/ml

QUALITY CONTROL PROCESS

- 5 During purification tests for protein content, protein purity, specific activity and pyrogen are conducted after each step. When the stock solution is obtained, all the tests listed in the table are done one after the other.
- 10 The quality of the product is controlled according to "Chinese Requirements for Biologics"
1. Original protein solution

Lowry

Item of Test	Method
Protein Stock Solution:	
Test for Protein Content	Lowry
Test for Protein Purity	Non-reductive SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) HPLC Analysis
Test for Molecular Weights	Reductive SDS-PAGE
Test for Specific Activity	According to Method in "Specific Activity Test of Interferon"
Test for Leftover Exogenetic DNA	Using DNA Labeling and Detection Kit
Test for Activity of Leftover Antibiotics	According to Method in "Chemical and Other Test Methods for Biologics"
Test for Bacterial Endotoxin	According to Method in "Requirements for Bacterial

Test for Isoelectronic Point	Endotoxin Test of Biologics" Isoelectric Focusing Electrophoresis
Test for Identify Characteristics of the Protein	UV spectrum (range of wavelength: 190-380nm)
	Peptide Mapping (hydrolyzed by pancreatic enzyme, analyzed by C-18 column)
	N-terminal Sequence Test
	C-terminal Sequence Test
	Circular Dichroism
	Amino Acid Analysis
Semi-finished Product	
Test for Bacterial Endotoxin	According to Method in "Requirements for Bacterial Endotoxin Test of Biologics"
Product	
Appearance Check	
Chemical	According to Method in "Chemical and Other Test Methods for Biologics"
Test for Specific Activity	According to Method in "Specific Activity Test of Interferon"
Sterility Test	According to Method in "c"
Abnormal Toxicity Test	Test on Mouse
Pyrogen Test	According to Method in "Requirements for Pyrogen Test of Biologics"
Test for Stability of Product	

Note: "Chemical and Other Test Methods for Biologics", "Requirements for Pyrogen Test of Biologics" and "Requirements for Bacterial Endotoxin Test of Biologics" all can be found in the "Chinese Requirements for Biologics." "Chinese Requirements for Biologics," PAN Zhengan, ZHANG Xinhui, DUAN Zhibing, et al. Chinese Biologics Standardization committee. Published by Chemical Industry Publishing Company, 2000.

EXAMPLE 3

Stability of lyophilized Powder of Recombinant Super-Compound Interferon Injection

5 The stability experiments were carried out with samples of lyophilized powder of recombinant super-compound interferon (rSIFN-co) injection in two specifications and three batches. The experiments started on April, 2000.

10 **1. Sample Source**

Samples were supplied by Sichuan Huiyang Life-engineering Ltd., Sichuan Province. Lot: 990101-03, 990101-05, 990102-03, 990102-05, 990103-03, 990103-05

15 **2. Sample Specifications**

Every sample in this experiment should conform with the requirements in the table below.

Table 1 Standard of Samples in Experiment

Items	Standards
1. Appearance	white loose powder
2. Dissolving time	dissolve rapidly in injection water(within 2 min) at room temperature
3. Clarity	colorless liquid or with little milk-like glisten; should not be cloudy, impurity or with indiscernible deposit
4. pH value	6.5~7.5
5. Potency (IU/dose)	80%~150% of indicated quantity (9μg:4.5 × 10 ⁶ IU, 15μg: 7.5 × 10 ⁶ IU)
6. Moisture	no more than 3.0% (w/w)

20 **3. Experiment Content**

15.3.1 Test samples at 2~8°C: The test samples were put into a 2~8°C refrigerator, then the above items of these samples were respectively tested in the 1st, 3rd, 6th, 9th, 12th, 18th, 24th, 30th, 36th month. The results were recorded.

15.3.2 Test samples at 25°C: The test samples were put into a thermostat at 25°C, then the above items of these samples were respectively tested in the 1st, 3rd, 6th, 9th, 12th, 18th, 24th, 30th month. The results were recorded.

5

15.3.3 Test samples at 37°C: The test samples were put into a thermostat at 37°C, then the above items of these samples were respectively tested in the 1st, 3rd, 6th, 9th, 12th, 18th, 24th month. The results were recorded.

10

4. Results and Conclusion

15 1) At 37°C, according to data collected at designated points during testing and compared with data before testing, the potency began descending from the 6th month and the changes in the three batches were similar. The appearance of other items had no changes.

20 2) At 25°C, according to data collected at designated points during testing and compared with data before the testing, the potency only had a little change, and the changes in the three batches were similar. The appearance of other items had no changes.

25 3). At 2-8°C, according to data collected at designated points during testing and compared with data before testing, the potency of the three batches all were stable. The appearance of other items also had no changes.

30 In conclusion, it is suggested that the lyophilized powder of recombinant super-compound interferon for injection should be better stored and transported at low temperatures. Without such conditions, the product can also be stored for short periods (i.e. 3 months) at room temperature.

EXAMPLE 4

35 rSIFN-co inhibits HBV-DNA duplication and secretion of HBsAg and HBeAg.

Materials

Solvent and Dispensing Method: Add 1ml saline into each vial, dissolve, and mix with MEM culture medium at 5 different concentrations. Mix on the spot.

Control drugs: IFN- α 2b (Intron A) as lyophilized powder, purchased from Schering Plough. 3×10^6 U each, mix to 3×10^6 IU/ml with culture medium; Infergen® (liquid solution), purchased from Amgen, 9ug, 0.3ml each, equal to 9×10^6 IU, and mix with 9×10^6 IU/ml culture medium preserve at 4°C; 2.2.15 cell: 2.2.15 cell line of hepatoma (Hep G2) cloned and transfected by HBV DNA, constructed by Mount Sinai Medical Center.

Reagent: MEM powder, Gibco American Ltd. cattle fetal blood serum, HycloneLab American Ltd. G-418(Geneticin); MEM dispensing, Gibco American Ltd.; L-Glutamyl, imported and packaged by JING KE Chemical Ltd.; HBsAg and HBeAg solid-phase radioimmunoassay box, Northward Reagent Institute of Chinese Isotope Ltd.; Biograncetina, Northern China Medicine; And Lipofectin, Gibco American Ltd.

Experimental goods and equipment: culture bottle, Denmark Tunclon™; 24-well and 96-well culture board, Corning American Ltd.; Carbon Dioxide hatching box, Shel-Lab American Ltd.; MEM culture medium 100ml: 10% cattle fetal blood serum, 3% Glutamyl1%, G418 380 μ g/ml, biograncetina50U/ml.

Method:

2.2.15 cell culture: Added 0.25% pancreatic enzyme into culture box with full of 2.2.15 cell, digest at 37°C for 3 minutes, and add culture medium to stop digest and disturb it to disperse the cells, reproduce with ratio of 1:3. They will reach full growth in 10 days.

Toxicity test: Set groups of different concentrations and a

control group in which cell is not acted on with medicine. Digest cell, and dispense to a 100,000 cell/ml solution. Inoculate to 96-well culture board, 200 μ l each well, culture at 37°C for 24h with 5% CO₂. Test when simple cell layer grows.

Dispense rSIFN-co to 1.8×10^7 IU/ml solution than prepare a series of solutions diluted at two-fold gradients. Add into 96-well culture board, 3 wells per concentration. Change the solution every 4 days. Test cytopathic effect by microscope after 8 days. Fully destroy as 4, 75% as 3, 50% as 2, 25% as 1, zero as 0. Calculate average cell lesion and inhibition rate of different concentrations. Calculate TC50 and TC0 according to the Reed Muench method.

15

$$TC50 = \text{Antilog} (B + \frac{50-B}{A-B} \times C)$$

A=log >50 % medicine concentration, B=log<50 %medicine concentration, C=log dilution power

20 Inhibition test for HBeAg and HBsAg: Separate into positive and negative HBeAg and HBsAg contrast groups, cell contrast group and medicine concentration groups. Inoculate 700,000 cells/ml of 2.2.15 cell into 6-well culture board, 3 ml each well, culture at 37°C for 24h with 5% CO₂, then prepare 25 5 gradiently diluted solutions with 3-fold as the grade (Prepare 5 solutions, each with a different protein concentration. The concentration of Solution 2 is 3 times lower than that of Solution 1, the concentration of Solution 3 is 3 times lower than that of Solution 2, etc.) 30 4.5×10^6 IU/ml, 1.5×10^6 IU/ml, 0.5×10^6 IU/ml, 0.17×10^6 IU/ml, and 0.056×10^6 IU/ml, 1 well per concentration, culture at 37°C for 24h with 5% CO₂. Change solutions every 4 days using the same solution. Collect all culture medium on the 8th day. Preserve at -20°C Repeat test 3 times to estimate HBsAg and 35 HBeAg with solid-phase radioimmunoassay box (Northward Reagent Institute of Chinese Isotope Ltd.). Estimate cpm

value of each well with a γ - accounting machine.

Effects calculation: Calculate cpm mean value of contrast groups and different-concentration groups and their standard deviation, P/N value such as inhibition rate, IC50 and SI.

5 1) Antigen inhibition rate (%) = $\frac{A-B}{A} \times 100$

A = cpm of control group; B = cpm of test group;

10 2) Counting the half-efficiency concentration of the medicine

$$\text{Antigen inhibition IC50} = \text{Antilog} (B + \frac{50-B}{A-B} \times C)$$

A=log>50% medicine concentration, B=log<50 % medicine concentration, C=log dilution power

15 3) SI of interspace-conformation changed rSIFN-co effect on HBsAg and HBeAg in 2.2.15 cell culture:

$$\text{SI} = \frac{\text{TC50}}{\text{IC50}}$$

20 4) Estimate the differences in cpm of each dilution degree from the control group using student t test

Southern blot: (1) HBV-DNA extract in 2.2.15 cell: Culture cell 8 days. Exsuction culture medium (Separate cells from culture medium by means of draining the culture medium.).
25 Add lysis buffer to break cells, then extract 2 times with a mixture of phenol, chloroform and isoamyl alcohol (1:1:1), 10,000g centrifuge. Collect the supernatant adding anhydrous alcohol to deposit nucleic acid. Vacuum draw, re-dissolve into 20 μ lTE buffer. (2) Electrophoresis: Add 6XDNA loading buffer, electrophoresis on 1.5% agarose gel, IV/cm, at fixed pressure for 14-18h. (3) Denaturation and hybridization: respectively dip gel into HCl, denaturaion buffer and neutralization buffer. (4) Transmembrane: Make an orderly transfer of DNA to Hybond-N membrane. Bake,

hybridize and expose with dot blot hybridization. Scan and analyze relative density with gel-pro software. Calculate inhibition rate and IC50.

5 **Results**

Results from Tables 1, 2 and 3 show: After maximum innocuous concentration exponent culturing for 8 days with 2.2.15 cell, the maxima is $9.0 \pm 0 \times 10^6$ IU/ml average inhibition rate of maximum innocuous concentration rSIFN-co 10 to HBeAg is $46.0 \pm 5.25\%$ ($P < 0.001$), IC50 is $4.54 \pm 1.32 \times 10^6$ IU/ml, SI is 3.96; rate to HBsAg is $44.8 \pm 6.6\%$, IC50 is $6.49 \pm 0.42 \times 10^6$ IU/ml, SI is 2.77. This shows that rSIFN-co can significantly inhibit the activity of HBeAg and HBsAg, but that the IFN of the contrast group and Infergen[®] cannot. It has also been proved in clinic that rSIFN-co can decrease HBeAg and HBsAg or return them to normal levels.

Table 1: Results of inhibition rate of rSIFN-co to HBsAg and HBeAg
First batch: (rSIFN-co)

Inhibition effect to HBeAg									
Concentration ($\times 10^4$ IU/ml)	First well	Seco nd well	Thir d well	Inhibition rate			Average inhibitio n rate	Accumulatio n	1- Accumulatio n
				First well	Second well	Third well			
900	9026	8976	1047	0.436227	0.43935	0.34565	0.407079	0.945909	0.592921
300	9616	12082	1009	0.399375	0.24534	0.36926	0.337997	0.5388299	1.254924
100	9822	16002	1280	0.386508	0.0005	0.2005	0.195836	0.200833	2.059088
33.33333	1577	19306	1682	0.014991	0	0	0.004997	0.0049969	3.054091
11.11111	1917	22270	1893	0	0	0	0	0	4.054091
Control	Cell	16010	Blank	0			Dilution	3	IC50 6
Inhibition effect to HBsAg									
Concentration ($\times 10^4$ IU/ml)	First well	Seco nd well	Thir d well	Inhibition rate			Average inhibitio n rate	Accumulatio n	1- Accumulatio n
				First well	Second well	Third well			
900	7706	7240	7114	0.342155	0.38193	0.39269	0.372261	0.922258	0.627739
300	8856	7778	9476	0.243981	0.33600	0.19105	0.257014	0.5499972	1.370724
100	1081	10720	1033	0.07649	0.08485	0.11814	0.093165	0.292983	2.27756
33.33333	1074	11114	1057	0.082807	0.05122	0.09766	0.07723	0.1998179	3.20033
11.11111	1067	9352	1081	0.088953	0.20163	0.07717	0.122588	0.122588	4.077742
Control	Cell	11714	Blank	0			Dilution	3	IC50 6

Second batch: (rsIFN-co)

Inhibition effect to HBeAg							
Concentration ($\times 10^4$ IU/ml)	First well	Second well	Third well	Inhibition rate		Average inhibition rate	1- Accumulatio n
				First well	Second well		
900	7818	8516	9350	0.554378	0.51459	0.46705	0.512008
300	1034	10628	9160	0.410396	0.39420	0.47788	0.427497
100	1229	14228	1326	0.299134	0.18901	0.24407	0.244072
33.33333	1536	17414	1618	0.124259	0.00741	0.77291	0.069653
11.11111	1738	13632	1540	0.009006	0.22298	0.12186	0.117951
Control	Cell	16962	Blank	0		Dilution	3
						IC50	365.935784
							6
Inhibition effect to HBsAg							
Concentration ($\times 10^4$ IU/ml)	First well	Second well	Third well	Inhibition rate		Average inhibition rate	1- Accumulatio n
				First well	Second well		
900	5784	6198	5792	0.498265	0.46235	0.49757	0.486063
300	7150	8534	8318	0.379771	0.25971	0.27845	0.30598
100	9830	11212	1021	0.147294	0.02741	0.11433	0.096345
33.33333	1394	12368	1347	0	0	0	0.0050891
11.11111	1241	11634	1135	0	0	0.01526	0.005089
Control	Cell		Blank	0		Dilution	3
						IC50	611.091956
							8

Third batch: (rSIFN-co)

Inhibition effect to HBeAg									
Concentration ($\times 10^4$ TU/ml)	First well	Second well	Third well	Inhibition rate			Accumulatio n	1- Accumulat ion	Accumulated inhibition rate
				First well	Second well	Third well			
900	9702	9614	8110	0.428016	0.433204	0.52187	0.461031	1.316983	0.538969
300	8914	10032	8870	0.4744723	0.40856	0.47706	0.453366	0.8559525	1.085603
100	16312	12688	13934	0.038321	0.251975	0.17851	0.156271	0.402586	1.929332
33.33333	15080	12814	13288	0.110954	0.244547	0.21660	0.190701	0.2463153	2.738631
11.11111	21928	15366	15728	0	0.094093	0.07275	0.0055615	0.055615	3.683017
Control	Cell	17544	Blank	0	Dilution	3	Dilution	3	IC50
Inhibition effect to HBsAg									
Concentration ($\times 10^4$ TU/ml)	First well	Second well	Third well	Inhibition rate			Accumulatio n	1- Accumulat ion	Accumulated inhibition rate
				First well	Second well	Third well			
900	5616	6228	5346	0.496864	0.442035	0.52105	0.486651	0.763125	0.513349
300	8542	8590	7096	0.234725	0.230425	0.36427	0.276474	0.2764738	1.236875
100	11420	11360	11394	0	0	0	0	0	2.236875
33.33333	12656	11582	13110	0	0	0	0	0	0
11.11111	13142	12336	13342	0	0	0	0	0	4.236875
Control	Cell	11528	Blank	0	Dilution	3	Dilution	3	IC50

HBeAg: Average IC50: 450.2434 SD: 132.315479

HBsAg: Average IC50: 649.1894 SD: 42.295880

HBeAg: Average IC50: 450.2434 SD: 132.315479

HBsAg: Average IC50: 649.1894 SD: 42.295880

Table 2: Results of inhibition rate of Intron A(IFN- α 2b) to HBsAg and HBeAg

Inhibition effect to HBeAg									
Concentration ($\times 10^4$ TU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1- Accumulation
				First well	Second well	Third well			
300	14918	11724	9950	0	0.029711	0.176529	0.068747	0.931253	0.068746724
100	14868	16890	15182	0	0	0	0	1.931253	0
33.33333	16760	21716	16400	0	0	0	0	2.931253	0
11.11111	20854	15042	16168	0	0	0	0	3.931253	0
3.703704	12083	12083	12083	0	0	0	0	4.931253	0
Control	Cell	17544	Blank	0	Dilution	3	IC50	FALSE	
Inhibition effect to HBsAg									
Concentration ($\times 10^4$ TU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibition rate	Accumulation	1- Accumulation
				First well	Second well	Third well			
300	9226	8196	9658	0.152489	0.247106	0.521054	0.1708	0.189295	0.8292
100	10946	10340	10828	0	0.050156	0.364272	0.018495	0.0184947	1.810705
33.33333	12250	12980	13934	0	0	0	0	0	0.010110817
11.11111	12634	12342	12000	0	0	0	0	0	3.810705
3.703704	10886	10886	10886	0	0	0	0	0	4.810705
Control	Cell	10886	Blank	0	Dilution	3	IC50	FALSE	

Table 3: Results of inhibition rate of Infergen® to HBsAg and HBeAg
First batch: (Infergen®)

Inhibition effect to HBeAg													
Concentration n ($\times 10^4$ IU/ml)	First well			Second well			Third well			Average inhibition rate	Accumulation n	1-Accumulation n	Accumulate d inhibition rate
	First well	Second well	Third well	First well	Second well	Third well	First well	Second well	Third well				
900 2	1417	12156	17306	0.091655	0.22086	0	0.104175	0.306157	0.895825	0.25471027	4		
300 0	1339	12288	16252	0.141776	0.21240	0	0.118062	0.2019827	1.777764	0.10202451	9		
100 4	1436	18834	14194	0.079349	0	0.09024	0.056531	0.083921	2.721232	0.02991667	8		
33.33333 2	1572	16034	16340	0	0	0	0	0.0273897	3.721232	0.00730659	2		
11.11111 4	1750	17652	14320	0	0	0.08216	0.02739	0.02739	4.693843	0.00580137	7		
Control Cell	Cell	15602	Blank	0			Dilution	3	IC50	FALSE			
Inhibition effect to HBsAg													
Concentration n ($\times 10^4$ IU/ml)	First well			Second well			Third well			Average inhibition rate	Accumulation n	1-Accumulation n	Accumulate d inhibition rate
	First well	Second well	Third well	First well	Second well	Third well	First well	Second well	Third well				
900 0	1208	11692	12234	0	0.01275	0	0.00425	0.025163	0.99575	0.02464711	1		
300 0	1284	11484	12350	0	0.03031	0	0.010104	0.0209125	1.985646	0.01042207	3		
100 4	1289	14696	15086	0	0	0	0	0.010808	2.985646	0.00360695	5		
33.33333 2	1503	12928	13020	0	0	0	0	0.0108081	3.985646	0.00270441	6		
11.11111 4	1179	11984	11508	0.004137	0	0.02828	0.010808	0.010808	4.974837	0.00216783	8		
Control Cell	Cell	11843	Blank	0			Dilution	3	IC50	FALSE			

Second batch: (Infergen®)

Inhibition effect to RBeAg													
Concentration ($\times 10^4$ IU/ml)	First well			Second well			Third well			Average inhibition rate	Accumulation n	1- Accumulation n	Accumulate d inhibition rate
	Firs t well	Secon d well	Thir d well	Firs t well	Secon d well	Third well	Firs t well	Secon d well	Third well				
900	6278	6376	6408	0.200051	0.18756	0.18348	0.190367	0.274635	0.0842678	0.274635	0.809633	0.25329050	5
300	7692	9092	6394	0.019877	0	0.18527	0.068383	0.015885	0.015885	0.015885	1.74125	0.04616100	5
100	8960	7474	8190	0	0.04765	0	0	0	0	0	2.725365	0.00579485	6
33.33333	8530	8144	9682	0	0	0	0	0	0	0	3.725365	0	0
11.11111	7848	7848	7848	0	0	0	0	0	0	0	4.725365	0	0
Control	Cell	7848	Blank	0	Dilution	3	IC50	IC50	FALSE				
Inhibition effect to HBsAg													
Concentration ($\times 10^4$ IU/ml)	First well			Second well			Third well			Average inhibition rate	Accumulation n	1- Accumulation n	Accumulate d inhibition rate
	Firs t well	Secon d well	Thir d well	Firs t well	Secon d well	Third well	Firs t well	Secon d well	Third well				
900	1236	12268	1227	0.036171	0.04365	0.04318	0.041004	0.140162	0.041004	0.041004	0.958996	0.12751773	7
300	1159	12708	1371	0.096507	0.00935	0	0.035287	0.0991581	0.035287	0.035287	1.923709	0.0490186	6
100	1244	13468	1398	0.029623	0	0	0.009874	0.063871	0.009874	0.009874	2.913834	0.02144964	5
33.33333	1261	11346	1244	0.016526	0.11552	0.02993	0.053996	0.053996	0.053996	0.053996	3.859838	0.01379630	9
11.11111	1282	12828	1282	0	0	0	0	0	0	0	4.859838	0	0
Control	Cell	12828	Blank	0	Dilution	3	IC50	IC50	FALSE				

Third batch: (Infergen®)

Inhibition effect to HBeAg									
Concentration ($\times 10^4$ IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibitio n rate	Accumulati on	1- Accumulati on
				First well	Second well	Third well			
900	7240	6642	6158	0.06459	0.1418	0.20439	0.136951	0.217399	0.863049
300	11072	8786	6902	0	0	0	0.10826	0.03609	0.0804479
100	7016	9726	7552	0.09354	0	0	0.02428	0.039276	0.044358
33.33333	7622	8866	8676	0.01524	0	0	0.005082	0.0050818	0.0050818
11.11111	7740	7740	7740	0	0	0	0	0	0
Control	Cell	7740	Blank	0	Dilution	3	IC50	FALSE	
Inhibition effect to HBsAg									
Concentration ($\times 10^4$ IU/ml)	First well	Second well	Third well	Inhibition rate			Average inhibitio n rate	Accumulati on	1- Accumulati on
				First well	Second well	Third well			
900	11048	11856	11902	0.04775	0	0	0.015917	0.015917	0.984083
300	13454	12896	11798	0	0	0	0	0	1.984083
100	12846	13160	12546	0	0	0	0	0	2.984083
33.33333	12680	12458	12360	0	0	0	0	0	3.984083
11.11111	11602	11602	11602	0	0	0	0	0	4.984083
Control	Cell	11602	Blank	0	Dilution	3	IC50	FALSE	

HBeAg: Average IC50: 0 SD: 0
 HBsAg: Average IC50: 0 SD: 0

EXAMPLE 5

Preparation of rSIFN-co

Preparation of lyophilized injection

Lyophilized powder

Stock Solution of 34.5 $\mu\text{g}/\text{ml}$

rSIFN-co

PB (pH7.0) 10mmol/L

Glycine 0.4mol/L

5

Preparation technique: Weigh materials according to recipe. Dissolve with sterile and pyrogen-free water. Filter through 0.22 μm membrane to de-bacterialize, preserve at 6-10°C. Fill in vials after affirming it is sterile and pyrogen-free, 0.3 ml /vial or 0.5 ml/vial, and lyophilize in freeze dryer.

Preparation of liquid injection

Solution

Stock Solution of 34.5 $\mu\text{g}/\text{ml}$

rSIFN-co

PB (pH7.0) 25mmol/L

NaCl 0.1mol/L

15

Preparation: Weigh materials according to recipe. Add to desired level with sterile and pyrogen-free water. Filter through 0.22 μm membrane to de-bacterialize, preserve at 6-10°C. Fill in airtight vial after affirming it is sterile and non-pyrogen at 0.3 ml /vial or 0.5 ml/vial. Storage at 2-10°C, and protect from light.

EXAMPLE 6

Acute Toxicity of rSIFN-co

25

Treat mice with large dose (150 $\mu\text{g}/\text{kg}$, equal to 1000 times of the normal dose per kilo used in treatment of adult patients) of rSIFN-co at one time by intramuscular injection. Then, observe and record their deaths and toxic reactions. Results show that: 24 hours after injection, no

abnormal reaction had been recorded. The organs of the animals which had been selected to be killed also had no signs of abnormal changes. Those remaining mice were all kept alive and were normal after two weeks. The weights of
5 mice in the experimental group and control group all increased, and the ratio of increase had no obvious difference between the two groups ($P>0.05$) according to their weights on the fourteenth day. No abnormal changes were seen from the main organs of those mice after two
10 weeks.

1. Experimental material

1.1 Animals

15 40 healthy adult mice, weighing 18-22g, half male and half female, qualified by Sichuan experiment animal control center.

2.2 Medicines

15 rSIFN-co (Provided by Sichuan Huiyang Life-engineering Ltd.) sterilized solution, 0.15 mg/ml, Lot: 981201
20 rSIFN-co was administered *i.m.* in saline.

2. Method

Separate the 40 mice into two groups randomly, one for experimental medicine, another for control. Inject
25 medicines or saline at the same ratio (0.1 ml/10 g) through muscle to each mouse according to which group they belong. (150 μ g/kg of rSIFN-co for experimental group; and saline for control group). After injection, observe and record acute toxicity shown in mice. Kill half of the mice (male
30 and female each half) to check whether there were any abnormal pathologic changes in their main organs, such as heart, spleen, liver, lung, kidney, adrenal gland, stomach, duodenum, etc. after 24 hours. Those remains were kept and observed until the fourteenth day. Weigh all mice, kill
35 them, and then observe the appearance of the organs listed above to see if there are any abnormalities. Take pathological tissue and examine it, using the examination to assess the difference in weight increases in the two

groups.

3. Results

Results show that there was no acute toxicity seen after
5 all mice were treated with i.m. rSIFN-co with 150 µg/kg at
a time, equal to 1000 times the normal dose per kilo used
in treatment of adult patients. In the 14 days after
injection, all mice lived well. They ate, drank, exercised,
and excreted normally and showed normal hair conditions.
10 None of them died. The observation of the main organs of
the randomly selected mice shows no abnormal changes 24
hours after injection. 14 days after injection, all
remaining mice were killed. Autopsies also showed no
changes. The weights of mice in the two groups all
15 increased, but no obvious difference was shown when
accessed with statistic method ($p > 0.05$). See Table 1:

Table 1 Influence to weights of mice after injection of
rSIFN-co

Group	Dose	Animal	Weights before injection (g)	Weights after injection (g)	Increased value of weights (g)
Control	0	20	19.8 ± 1.7	30.8 ± 2.8	11.0 ± 2.9
rSIFN-co	150	20	19.4 ± 1.7	32.1 ± 3.3	12.7 ± 4.3

20

3. Conclusion

Under conditions of this experiment, there were no toxic reactions in all mice after injection of rSIFN-co with 150 µg/kg. The conclusion can be reached that the maximum tolerable dose of i.m. in mice is 150 µg/kg, which is equal to 1000 times the normal dose per kilo used in treatment of adult patients.

EXAMPLE 7

30 The clinic effects of recombinant super-compound interferon (rSIFN-co)

The recombinant super-compound interferon (rSIFN-co) is an

invention for viral disease therapy, especially for hepatitis. Meanwhile, it can inhibit the activity of EB viruses, VSV, Herpes simplex viruses, coronaviruses, measles viruses et al. Using Wish cells /VSV system as the assay for anti-virus activity, the results showed that: the other rIFN, was 0.9×10^8 IU/mg, Intron A was 2.0×10^8 IU/mg and rSIFN-co was 9×10^8 IU/mg. The anti-viral activity of rSIFN-co is much higher than those of the former two.

Under the permission of the State Food and Drug Administration (SFDA), People's Republic of China, the clinical trials have taken place in West China Hospital, Sichuan University, the Second Hospital of Chongqing Medical University, the First Hospital of School of Medical, Zhejiang University since the Feb 2003. The clinical treatment which focuses on the hepatitis B is conducted under the guidance of the multilcenter, double-blind random test. IFN- α lb was used as control, and the primary results showed the following:

The effect of rSIFN-co compared with IFN- α lb in the treatment of chronic active hepatitis B

1. Standard of patients selection: The standard 1-4 are effective to both treatment with rSIFN-co ($9\mu\text{g}$) and IFN- α lb (5MU, $50\mu\text{g}$), and the standard 1-5 are for rSIFN-co ($15\mu\text{g}$) treatment.

- 1). Age: 18-65
- 2). HBsAg test positive last over six months, HBeAg test positive, PCR assay, HBV-DNA copies $\geq 10^5/\text{ml}$
- 3). ALT \geq two times of the normal value
- 4). Never received IFN treatment; or those received the Lamividine treatment but failed or relapsed
- 5) Once received other IFNs (3MU or 5MU) treatment six months ago, following the standard of SFDA but failed or relapsed

2. Evaluation of the effects:

In reference to the recommendations from the Tenth China National Committee of Virus Hepatitis and Hepatopathy, the effects were divided into three degrees according to the ALT level, HBV-DNA and HBeAg tests.

Response: ALT normal level, HBV-DNA negative, HBeAg negative

Partial response: ALT normal level, HBV-DNA or HBeAg negative

Non response: ALT, HBV-DNA and HBeAg unchanged

The response and partial response groups consider as effective cases.

3. Results of clinic trial:

Group A: treatment with rSIFN-co(9μg)

Group B: treatment with IFN-αlb (5MU, 50 μg)

Period	group	Medicine	cases	Effecti ve Rate	HBsAg Transfe r to negativ e rate	HBeAg Transfe r to negativ e rate	HBV-DNA Transfe r to negativ e rate	Heptal functio n Recover rate
8-12 week	A	rSIFN-co(9μg)	32	46.88 (15)	9.38 (3)	28.12 (9)	37.50 (12)	84.38 (27)
	B	IFN-αlb (5MU, 50 μg)	32	21.88 (7)	0.00 (0)	9.38 (3)	15.62 (5)	56.25 (18)
16-24 week	A	rSIFN-co(9μg)	64	54.69 (35)	7.81 (5)	25.00 (16)	34.38 (22)	90.62 (58)
	B	IFN-αlb (5MU, 50 μg)	64	25.00 (16)	0.00 (0)	9.38 (6)	18.75 (12)	78.13 (50)

In Group C, the cases were chronic active hepatitis B treatment with other IFNs (3MU or 5MU) before but failed or relapsed and treated with rSIFN-co (15 μg), subcutaneous injection, every one day, last 24 weeks. The total cases are

13. After 12 weeks treatment, 7 of 13 (53.85%) were effective. 3 of 13 (23.08%) HBeAg transferred to negative; 7 of 13(53.85%) HBV-DNA transferred to negative; 11 of 13 (84.62%) hepatal functions recovered to normal.

4. The side effects of rSIFN-co compared with IFN-αlb in the treatment

The side effects of IFN include fever, nausea, myalgia, anorexia, hair lose, leucopenia and thrombocytopenia, etc. The maximum dose of IFN- α 1b is 5MIU per time; the routine dose is 3 MIU. When taken the routine dose, 90% patients have I- II degree (WHO standard) side effects. They are fever lower than 38°C, nausea, myalgia, anorexia, etc. When taken at maximum dose, the rate of side effects do not rise obviously, but are more serious. The maximum dose of rSIFN-co is 24 μ g, subcutaneous injection, every one day for 3 months. The routine dose is 9 μ g. When routine doses were used, less than 50% patients have I-II degree (WHO standard) side effects, including fever below 38°C, nausea, myalgia, anorexia, leucopenia and thrombocytopenia slightly. With maximum dosage, about 50% patients suffered from leucopenia and thrombocytopenia after using rSIFN-co one month, but those side effects would disappear after stopping treatment for one week. It is safe for continue use.

The observations of rSIFN-co treat hepatitis C

1. Standard of patient's selection

- 1) age: 18-65
- 2) HCV antibody positive
- 3) ALT \geq 1.5 times of the normal value, last more than 6 months

2. Evaluation of the effects:

Referring to the standard of Infergen® for treatment of hepatitis C and according to the ALT level and HCV-RNA test, divided the effects into three degree:

Response: ALT normal level, HCV-RNA negative

Partial response: ALT normal level, HCV-RNA unchanged

Non response: ALT and HCV-RNA unchanged

3. Effects in clinic

The clinical trial was done at the same time with hepatitis B treatment. 46 cases received the treatment, 9 μ g each time, subcutaneous injection, every day for 24 weeks. After treatment, 26 of 46 (56.52%) have obvious effects, 12 of 46

(26.08%) HCV-RNA transferred to negative, 26 of 46 (56.52%)
hepal functions recovered to normal.